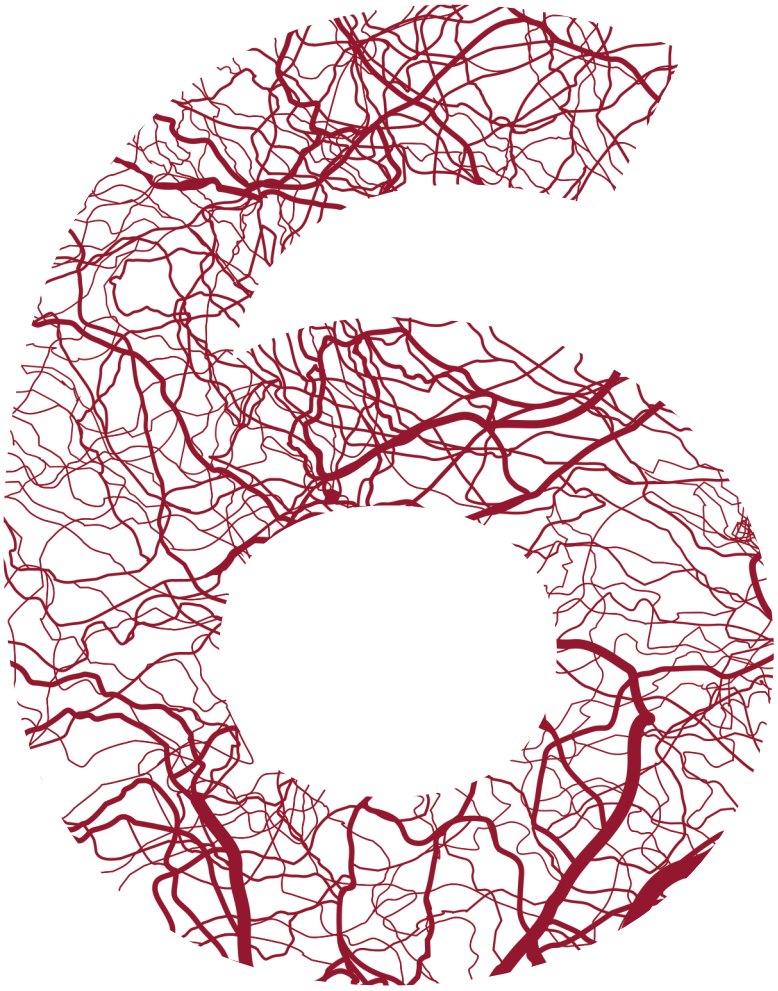


# CHAPTER 6

# Partial deletion of Tie2 affects microvascular endothelial responses to critical illness in a vascular bed and organ-specific way

Jongman RM, Zwiers PJ, van de Sluis B, van der Laan M, Moser J, Zijlstra J. G, Dekker D, Huijkman N, Moorlag HE, Popa ER, Molema G, van Meurs M.

**Submitted.**

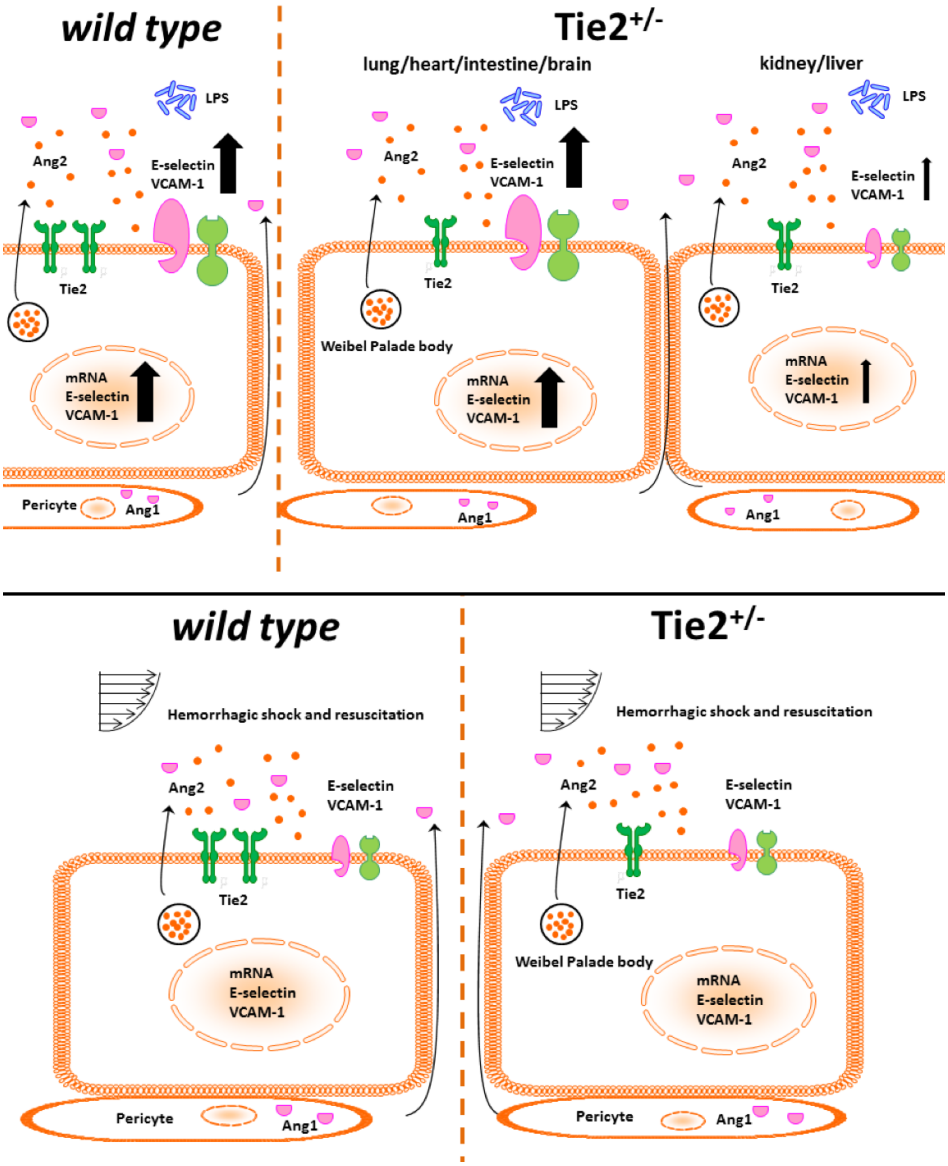
## Abstract

**Objective:** Tie2 is a tyrosine kinase receptor that is mainly expressed by endothelial cells. In animal models mimicking critical illness, Tie2 levels in organs are temporarily reduced. Functional consequences of these reduced Tie2 levels on microvascular endothelial behavior are important to understand. We investigated the effect of partial deletion of Tie2 on the activation status of endothelial cells in different organs.

**Approach and results:** Newly-generated heterozygous Tie2 knockout mice (exon 9 deletion, ΔE9/Tie2$^{+/-}$) exhibiting 50% reduction in Tie2 mRNA and protein, and *wild type* littermate controls (Tie2$^{+/+}$), were subjected to hemorrhagic shock and resuscitation (HS + R), or challenged with *i.p.* lipopolysaccharide (LPS). Kidney, liver, lung, heart, brain, and intestine were analyzed for mRNA levels of adhesion molecules E-selectin, VCAM-1, and ICAM-1, and CD45. Exposure to HS + R did not result in different expression responses of these molecules between organs from Tie2$^{+/-}$ or Tie2$^{+/+}$ mice and sham-operated mice. In contrast, the LPS-induced mRNA expression levels of E-selectin, VCAM-1, and ICAM-1, and CD45 in organs of Tie2$^{+/+}$ mice were attenuated in Tie2$^{+/-}$ mice when compared to Tie2$^{+/+}$ mice in kidney and liver, but not in the other organs studied. Furthermore, reduced expression of E-selectin and VCAM-1 protein, and reduced influx of CD45$^{+}$ cells upon LPS exposure, was visible in a microvascular bed-specific pattern in kidney and liver of Tie2$^{+/-}$ mice compared to controls.

**Conclusion:** Heterozygous deletion of Tie2, is associated with microvascular bed-specific attenuation of endothelial responses to LPS, implying a microvascular bed-specific role for Tie2 in inflammatory endothelial activation control.

## Graphical abstract



# Introduction

Tie2 is a receptor tyrosine kinase that is mainly expressed by endothelial cells [1]. Tie2 interacts with its ligands Angiopoietin (Ang) 1 and Ang2 to facilitate blood vessel development, and vessel stabilization or destabilization in mature vessels. In quiescent conditions of the mature vasculature, Ang1 binds to Tie2 leading to dimerization of the Tie2 receptor and subsequent activation of several intracellular pathways that maintain endothelial integrity [2].

In inflammatory conditions, the endothelium becomes activated and expresses adhesion molecules such as E-selectin, VCAM-1, and ICAM-1, which serve as guidance for leukocytes to move to the site of inflammation. Furthermore, Ang2 is secreted by activated endothelial cells to induce destabilization of the endothelium by competing with Ang1 for the Tie2 receptor, leading to increased vascular permeability [3]. Data also suggest the existence of a functional link between the Angiopoietins and the response of endothelial cells in inflammation [4, 5]. For example, adenoviral production of Ang1 inhibited *in vivo* leukocyte infiltration in a lipopolysaccharide (LPS)-induced endotoxemia mouse model [6]. Similarly, *in vitro* Ang1 treatment partially inhibited adhesion and trans-endothelial migration of leukocytes, which was accompanied by suppressed expression of adhesion molecules expression in endothelial cells [7, 8]. In Ang2 knockout mice, reduced leukocyte influx of neutrophils in response to *i.p* injection of bacteria was reported [9]. Moreover, *in vivo* blockade of Ang2 reduced infiltration of leukocytes and expression of adhesion molecules in the lung, and at the same time inhibited vascular remodeling [10].

While we understand the effects of concentration changes the ligands Ang1 and Ang2, we know little about the effects of changes in expression levels of the Tie2 receptor on the inflammatory response of the endothelium. Previously, we reported reduced expression of Tie2 in kidney biopsies of sepsis patients [11], as well as in organs of mice subjected to hemorrhagic shock (HS) and LPS-induced endotoxemia [12]. However, the functional consequences of this reduced expression for endothelial behavior were not explored. In the present study, our aim was to investigate the effects of partial deletion of Tie2 on endothelial responses in two animal models of critical illness, with focus on the microvasculature in the different organs

of these mice, as endothelial cells in different (micro)vascular beds were previously reported to respond differently to inflammatory stimuli [13-15].

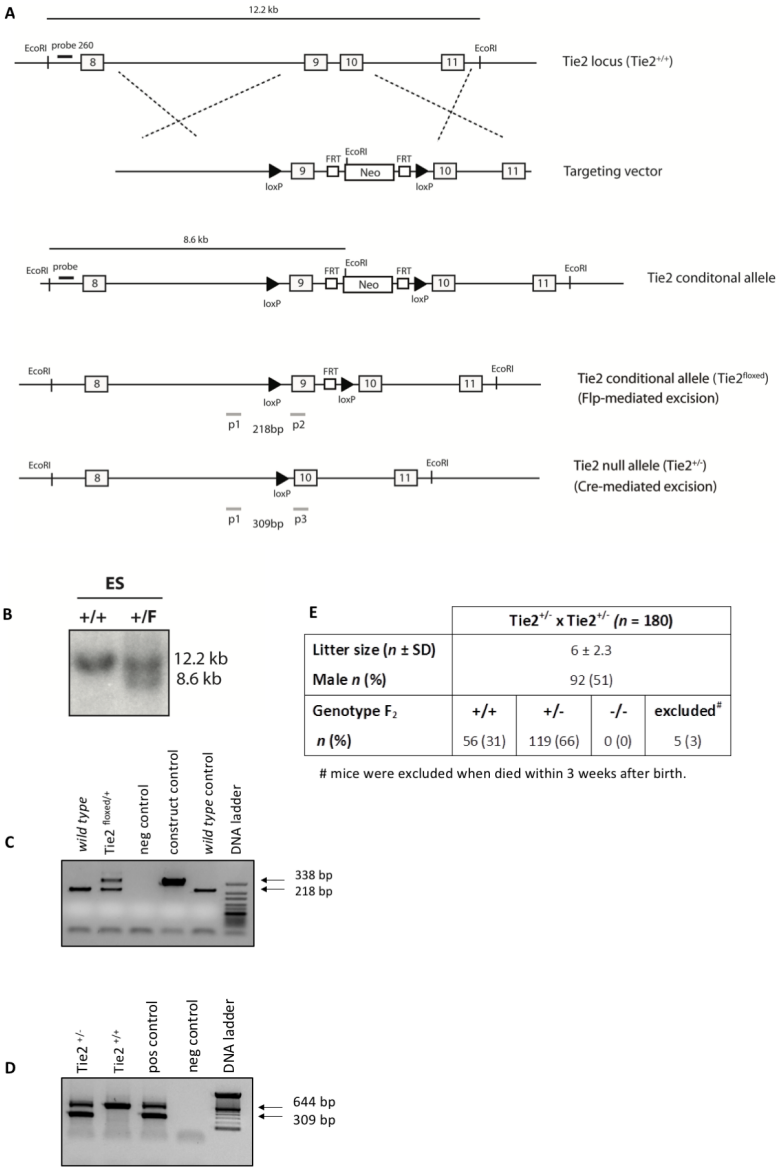
To this end, we created a condition of lower Tie2 expression by generating a heterozygous Tie2 knockout mouse model based on deletion of exon 9 (ΔE9/Tie2$^{+/-}$, hereafter referred to as Tie2$^{+/}$). We verified that these mice express 50% lower Tie2 protein compared to their *wild type* (WT) littermate controls, after which we investigated whether this genetically constructed reduction in Tie2 expression affected basal expression of the Tie2 ligands Ang1 and Ang2 and basal endothelial inflammatory status. We further examined the effects of hemorrhagic shock followed by resuscitation (HS + R), and of endotoxemia induction by *i.p.* LPS treatment on endothelial responses and leukocyte recruitment to the organs. We compared responses in Tie2$^{+/-}$ mice to those in *wild type* mice by studying whole organ responses as well as responses in specific microvascular segments in these organs.

# Materials and Methods

### Generation of heterozygous Tie2$^{+/-}$ mice

The Tie2$^{floxed}$ mouse line was generated by homologous recombination of the Tie2 allele using a method described previously [16]. Briefly, a genomic fragment (12.2 kb) of the Tie2 gene spanning exons 9-11 was obtained from BAC #bMQ279D1 (129S7/SvEv ES cell, Source BioScience) and cloned into the pDTA.4B vector. An orphan loxP site was inserted into the pDTA.4B-Tie2 (ex9-ex11) construct, 119 bp upstream of exon 9 using recombineering (Figure 1A). The *frt-neo-frt-loxP* cassette was inserted into the targeting construct 189bp downstream of exon 9. The final construct was linearized with *Apa*I and electroporated into TL1 129Sv/E ES cells. Subsequently the cells were selected in medium supplemented with G418, and expanded. Southern blot analysis was performed using a 198bp 5' external probe on *Eco*RI-digested genomic ES cell DNA (Figure 1B). Oligo-sequences used for recombineering and the Southern blot probe can be obtained upon request.

Chimeric mice were generated by microinjection of two independent ES cell targeted clones into C57BL/6 blastocysts. Chimeric males were mated with C57BL/6 females and germline transmission of the floxed Tie2 allele (Tie2$^{floxed-neo}$) was confirmed by PCR analysis using 5'-GCTCGACGTTGTCACTGAAG-3' and 5'-CCATTTTCCACCATGATATTCG-3' primers. The neo cassette was




E

| | Tie2$^{+/-}$ x Tie2$^{+/-}$ (*n* = 180) | | | |
|---|---|---|---|---|
| **Litter size (*n* ± SD)** | 6 ± 2.3 | | | |
| **Male *n* (%)** | 92 (51) | | | |
| **Genotype $F_2$** | **+/+** | **+/-** | **-/-** | **excluded#** |
| ***n* (%)** | 56 (31) | 119 (66) | 0 (0) | 5 (3) |

# mice were excluded when died within 3 weeks after birth.

**FIGURE 1. Generation of the Tie2$^{floxed}$ mouse line. A.** Schematic representation of the 12.2 kb genomic fragment of Tie2. LoxP sites were inserted up- and downstream of exon 9. p1, p2, and p3 represent binding sites for primers resulting in pcr products as described in C and D. **B.** Southern blot analysis using a 5' external probe on *EcoRI*-digested gDNA. +/+ wild type allele; +/F, floxed allele **C.** Genomic pcr analyses with primer p1 and p2 confirmed presence (338 bp, Tie2$^{floxed/+}$) or absence of loxP sites (218 bp, wild type). **D.** Tie2$^{floxed/floxed}$ male offspring crossed with Hprt-cre females produced a cre-mediated excision and resulted in a 309 bp (Tie2$^{+/-}$) product when exon 9 was excised and/or 644 bp (Tie2$^{+/+}$) product when exon 9 was present using primers p1 and p3. E. Characteristics and genotypes of the offspring of $F_1$ intercross Tie2$^{+/-}$ mice.

excised by breeding the Tie2$^{floxed\text{-}Neo}$ mice with mice expressing flippase recombinase (*ACTFLPe*, Jackson Laboratory, Bar Harbor, USA, strain #005703).

Mice carrying one Tie2 null allele (Tie2$^{+/-}$) were generated by crossing Tie2$^{floxed/floxed}$ mice with mice expressing Cre-recombinase in the female germline (*Hprt-Cre*, Jackson Laboratory, strain #004302). In this study, litters resulting from $F_1$ intercrossing of Tie2$^{+/-}$ mice were used.

## Genotyping

Mouse genomic DNA was extracted from ear punches using standard protocols. The genotype of Tie2$^{floxed}$ mice was determined by PCR analysis using 5'-GGCCACTGAGAAACGATCTG-3' and 5'-GGGCTGCTACAATAGCTTTGG-3' primers, resulting in a 338bp PCR product when *loxP* sites were present (Tie2$^{floxed/+}$) and in a 218bp PCR product when loxP sites were absent (Tie2$^{+/+}$) (Figure 1C).

The genotype of Tie2$^{+/-}$ mice was determined by PCR using the primers 5'-GGGCTGCTACAATAGCTTTGG-3' and 5'-GTTATGTCCAGTGTCAATCAC-3' resulting in a 644bp PCR product when exon 9 is still present (Tie2$^{+/+}$) and in a 309bp PCR product when exon 9 of Tie2 was excised by Cre-recombinase (Tie2$^{+/-}$) (Figure 1D). PCR products were run on a 1.5% (*w/v*) agarose gel in Tris-borate-EDTA-buffer with 0.005% (v/v) ethidium bromide, and visualized under UV-light.

## Mouse shock models

All animal experimentation was done according to institutional and national guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Groningen.

### *Hemorrhagic shock model*

Mouse hemorrhagic shock (HS) was induced as previously described [17]. Briefly, mice were anesthetized with isoflurane and kept on a temperature-controlled (37°C-38°C) surgical pad. HS was induced by blood withdrawal from the left femoral artery, until a reduction of the mean arterial pressure to 30 mmHg was reached. To maintain the mean arterial pressure at 30 mmHg, small volumes of blood were withdrawn or restituted during the shock period. After 90 min of shock, mice were resuscitated (HS+R) with

4% human albumin in saline (Sanquin, Amsterdam, the Netherlands) at two times the volume of blood withdrawn. Mice were sacrificed 1 hour after resuscitation. Sham-operated mice underwent instrumentation and were kept under anesthesia for the same period as HS mice, without withdrawal of blood. At sacrifice, blood was drawn via cardiac puncture and organs were harvested, snap-frozen on liquid nitrogen and stored at -80°C until analysis. Groups consisted of 6 mice each.

*Endotoxemia model*

To induce of endotoxemia, mice were intraperitoneally (*i.p.*) injected with 1 µg/g body weight lipopolysaccharide (LPS) (*E. coli*, serotype O26:B6, Sigma-Aldrich, St. Louis, MO, USA) in NaCl 0.9% (*w/v*). Vehicle control mice were. injected *i. p* with NaCl 0.9% (*w/v*). All mice were sacrificed under isoflurane/$O_2$ anesthesia 4 hours after LPS or vehicle administration. Blood was drawn via cardiac puncture and organs were harvested, snap-frozen on liquid nitrogen and stored at -80°C until analysis. Groups consisted of 6 mice each.

## RNA isolation and gene expression analysis by quantitative RT-PCR

To study gene expression levels, total RNA was isolated from tissues with the RNeasy® Plus Mini Kit, (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. RNA concentration ($OD_{260}$) and purity ($OD_{260}/OD_{280}$) was measured using an ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). RNA integrity was determined by gel electrophoresis.

cDNA was synthesized using random hexamer primers (Promega, Leiden, the Netherlands) and SuperScript III (Invitrogen, Breda, the Netherlands). Assay-on-Demand primers/probe sets (TaqMan® Gene Expression) were purchased from Thermo Fisher Scientific (Bleiswijk, the Netherlands) (Table 1). Duplicate quantitative PCR analyses were performed on ViiA7 Real-Time PCR system (Thermo Fisher Scientific) for each sample and the obtained threshold cycle values (CT) were averaged. Gene expression was normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase *(Gapdh)*, yielding the ΔCT value. The average mRNA level relative to GAPDH was calculated by $2^{-\Delta CT}$.

**TABLE 1: RT-qPCR primers.**

| Gene | Assay ID | Encoded protein |
|---|---|---|
| *Gapdh* | Mm99999915_g1 | Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) |
| *Tek* | Mm00443242_m1 | Tyrosine kinase receptor (Tie2), CD202 |
| *Angpt1* | Mm00456503_m1 | Angiopoietin 1 |
| *Angpt2* | Mm00545822_m1 | Angiopoietin 2 |
| *Sele* | Mm00441278_m1 | E-selectin, CD62E |
| *Vcam1* | Mm00449197_m1 | Vascular cell adhesion molecule 1 (VCAM-1), CD106 |
| *Icam1* | Mm00516023_m1 | Intercellular adhesion molecule 1 (ICAM-1), CD54 |
| *Ptprc* | Mm00448463_m1 | Protein tyrosine phosphatase receptor type C, CD45 |

## Protein quantification by ELISA

Tissue homogenates were prepared from cryosections of organs by lysis in RIPA buffer on ice (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% (*w/v*) sodium deoxycholate, 0.1% (*w/v*) SDS, 1% (*v/v*) IGEPAL) containing protease inhibitor (Roche Diagnostics, Almere, The Netherlands), phosphatase inhibitor (Roche) and 1 mM activated $Na_3VO_4$. Total protein concentration was determined by DC Protein Assay (Bio-Rad Laboratories, Veenendaal, The Netherlands).

Protein expression of Tie2 in organs was quantified by ELISA according to manufacturer's instructions (R&D Systems, Abingdon, UK). Tie2 Amounts were normalized for the total protein input of tissue homogenate and expressed as pg/µg total protein. Protein concentration of soluble Tie2 was measured in plasma using the same ELISA kit.

## Localization of proteins by immunohistochemistry

To study protein expression in different microvascular beds in organs, 4 µm cryosections were cut and fixed with acetone. After blocking endogenous peroxidase with 0.075% (*v/v*) $H_2O_2$ in PBS, sections were incubated for 1 hour at room temperature with primary antibodies for E-selectin (clone Mes-1, a kind gift from Dr. Brown, UCB Celltech, Brussels, Belgium), VCAM-1 (clone M/K-2, Merck Millipore, Amsterdam, The Netherlands), or CD45 (clone 30-F11, BD Biosciences, Breda, The Netherlands). All primary antibodies were diluted in PBS 5% (*v/v)* FCS (Sigma-Aldrich). Isotype controls IgG1, IgG2a and IgG2b (Antigenix America, New York, USA) were consistently found to be negative. Next, slides were incubated with secondary rabbit-anti-rat IgG antibody (Vector Laboratories, Burlingame, CA, USA) in PBS supplemented

with 5% (*v/v*) FCS and 1% (*v/v*) normal mouse serum (Sanquin) for 45 min, followed by anti-rabbit, HRP-labeled polymer (Dako Netherlands, Heverlee, Belgium) for 30 min. Between incubation steps, slides were washed extensively with PBS. Peroxidase activity was detected with 3-Amino-9-ethylcarbazole (Sigma-Aldrich). Sections were counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany).

Stained sections were scanned with NanoZoomer® 2.0 HT (Hamamatsu Photonics, Almere, The Netherlands). Immunohistochemical stainings were quantified using Aperio Imagescope software v12.1 (Leica Biosystems Imaging, Vista, CA, USA). Briefly, regions of interest were drawn around the perimeter of the tissue sections, excluding occasional artifacts (tissue breaks or folds). After automated counting of pixels, the ratio of positive pixels/total pixels was calculated. Next, the fold change of the ratio positive pixels/total pixels between LPS-challenged mice and their vehicle controls was calculated and plotted.

### Statistical analysis

To compare gene expression patterns between $Tie2^{+/-}$ mice and $Tie2^{+/+}$ mice and responses to HS + R or LPS, fold change of expression levels between HS+R and sham or between LPS-challenged mice and their vehicle controls was calculated as follows: average relative mRNA expression of the sham or vehicle treated $Tie2^{+/+}$ or $Tie2^{+/-}$ group was set at 1. Relative mRNA levels of individual HS + R or LPS-treated mice were divided by the average mRNA levels of their respective sham or vehicle group. Statistical significance between $Tie2^{+/+}$ and $Tie2^{+/-}$ mouse responses was evaluated by a two-tailed unpaired Student's *t*-test. Statistics were performed using GraphPad Prism 7.0 (GraphPad Prism Software Inc. La Jolla, CA, USA). Differences were considered to be statistically significant when $P<0.05$.

# Results

## Generation and characterization of Tie2$^{+/-}$ mice

We first constructed a Tie2$^{+/-}$ mouse line by the deletion of exon 9 of Tie2. Crossing homozygous Tie2$^{floxed/floxed}$ mice with *Hprt-Cre* mice resulted in 100% Tie2$^{+/-}$ offspring. $F_1$ intercrossing of Tie2$^{+/-}$ mice resulted in $F_2$ generations of which 66% were Tie2$^{+/-}$ mice, and 31% were Tie2$^{+/+}$. Tie2$^{-/-}$ mice were not born (Figure 1E).

To confirm that Tie2 levels were indeed reduced by 50% in the newly generated mouse line, we analyzed Tie2 expression levels in kidney, liver, lung, heart, brain, and intestine. In these organs, Tie2 mRNA and protein levels were approximately 50% lower in Tie2$^{+/-}$ mice than in Tie2$^{+/+}$ littermates (Figure 2).

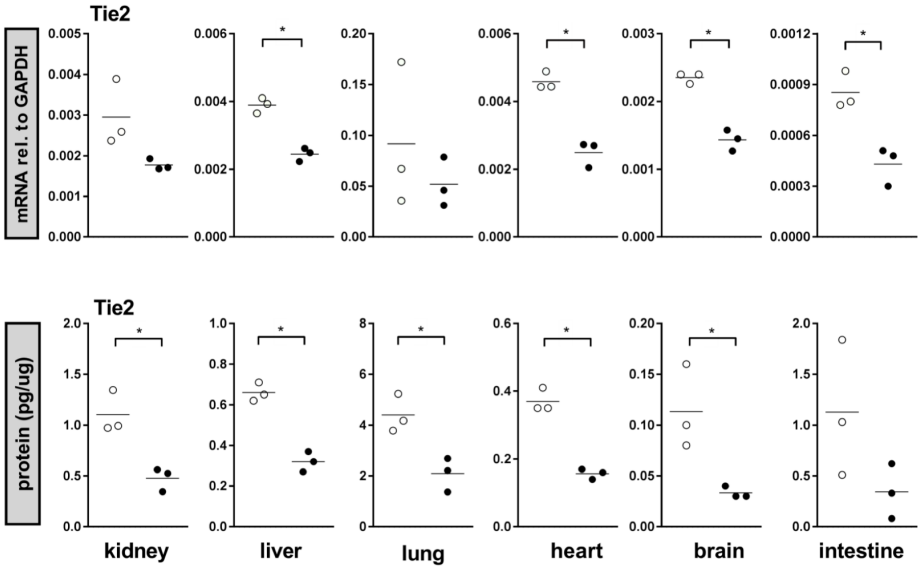



**FIGURE 2. Tie2 expression is reduced to half in organs of Tie2$^{+/-}$ mice.** Organs of Tie2$^{+/+}$ and Tie2$^{+/-}$ mice were assessed for mRNA and protein levels. **A.** Tie2 mRNA levels by RT-qPCR relative to GAPDH. **B.** Tie2 protein levels in organs as determined by ELISA. Dots represent individual Tie2$^{+/+}$ mice (o), Tie2$^{+/-}$ mice (●), horizontal lines indicate average values of 3 mice per group, * $P$<0.05.

Since Tie2 was reported to be expressed not only expressed by endothelial cells, but also, to a minimal extent, by hematopoietic cells [18], we analyzed Tie2 mRNA expression in total white blood cell isolates of $Tie2^{+/+}$ and $Tie2^{+/-}$ mice. Tie2 mRNA was not detectable in white blood cells of either mouse line, in contrast to the highly-expressed pan-leukocyte marker protein tyrosine phosphatase receptor type C *(Ptprc)* encoding CD45 protein (Suppl. Table 1). Thus, deletion of exon 9 of Tie2 from one allele resulted in a 50% reduction of Tie2 expression in the organs.

### Basal mRNA expression levels of Angiopoietins and genes related to endothelial inflammatory activation in $Tie2^{+/-}$ mice

Since Tie2 is constitutively expressed by endothelial cells, a reduction in its protein levels, as effected by partial knockout of the Tie2 gene at the start of life in embryo, may potentially result in adaptation of expression of its ligands Ang1 and Ang2. We found no differences in basal mRNA expression levels of Ang1 and Ang2 in kidney, liver, lung, heart, brain, and intestine between $Tie2^{+/-}$ mice and $Tie2^{+/+}$ mice, irrespective of the organ studied (Suppl. Figure 1A).

Next, we examined whether partial deletion of Tie2 has consequences for basal expression levels of the endothelial inflammatory activation genes E-selectin, VCAM-1, and ICAM-1 (Suppl. Figure 1B). In both mouse lines, basal expression of these genes showed organ-dependent differences. The highest expression of E-selectin, VCAM-1, and ICAM-1 was found in the lung, while the lowest expression of E-selectin and VCAM-1 was found in the brain, the lowest expression of ICAM-1 in intestine. No differences in basal gene expression were found between $Tie2^{+/-}$ and $Tie2^{+/+}$ mice in any of the organs. Since the studied adhesion molecules are mainly expressed by endothelial cells, we also investigated the expression levels of endothelial-restricted molecules platelet endothelial cell adhesion molecule 1 (CD31, *Pecam1*) and vascular endothelial cadherin (VE-cadherin, *Cdh5*). These varied between organs due to the differences in endothelial content between organs, yet did not differ between $Tie2^{+/-}$ and $Tie2^{+/+}$ mice in any of the organs studied (Suppl. Figure 2).

The endothelial adhesion molecules E-selectin, VCAM-1, and ICAM-1 participate in leukocyte adhesion and extravasation. Since partial deletion of Tie2 protein did not affect basal expression of these molecules, we

postulated that leukocyte recruitment for homeostatic surveillance purposes would also not be affected in quiescent organs. Indeed, mRNA of CD45 was detected in all organs and no differences between Tie2$^{+/-}$ and Tie2$^{+/+}$ mice were found in any of the organs analyzed (Suppl. Figure 1C).

In conclusion, reduction of Tie2 protein by 50% in Tie2$^{+/-}$ mice did not affect basal expression levels of its ligands Ang1 and Ang2. Moreover, basal expression levels of genes related to endothelial inflammatory activation, and the associated presence of leukocytes in the main organs, did not change.

## Endothelial responses to hemorrhagic shock in Tie2$^{+/-}$ mice

We next investigated whether partial deletion of Tie2 protein affected endothelial responses in two models of critical illness. For this, we first employed hemorrhagic shock followed by resuscitation (HS + R), a model of critical illness which systemically affects all organs [13]. We studied mRNA expression of endothelial adhesion molecules E-selectin, VCAM-1, and ICAM-1, and CD45 in kidney, liver, and lung, since we have previously shown that these organs are most extensively affected by HS + R [17].

HS + R led to a reduction of Tie2 mRNA levels in kidneys of *wild type* Tie2$^{+/+}$ mice, while lower Tie2 mRNA levels in liver and lung in this experiment were statistically not significantly different compared to sham controls (Suppl. Figure 3A). In Tie2$^{+/-}$ mice, in which Tie2 expression was already reduced by 50% at the start of HS induction, the extent of downregulation of Tie2 after HS + R in kidney, liver and lung was similar as in WT mice (Suppl. Figure 3B).

The expression of E-selectin, VCAM-1, and ICAM-1, was not affected by HS + R in either Tie2$^{+/+}$ or in Tie2$^{+/-}$ mice, irrespective of the organ (Figure 3A). Moreover, CD45 mRNA levels did not differ between HS + R treated mice and sham treated mice in any of the organs of either genotype (Figure 3B).

Summarizing, no changes in expression of genes related to endothelial activation and leukocyte influx could be observed between Tie2$^{+/-}$ and Tie2$^{+/+}$ mice when exposed to HS + R.

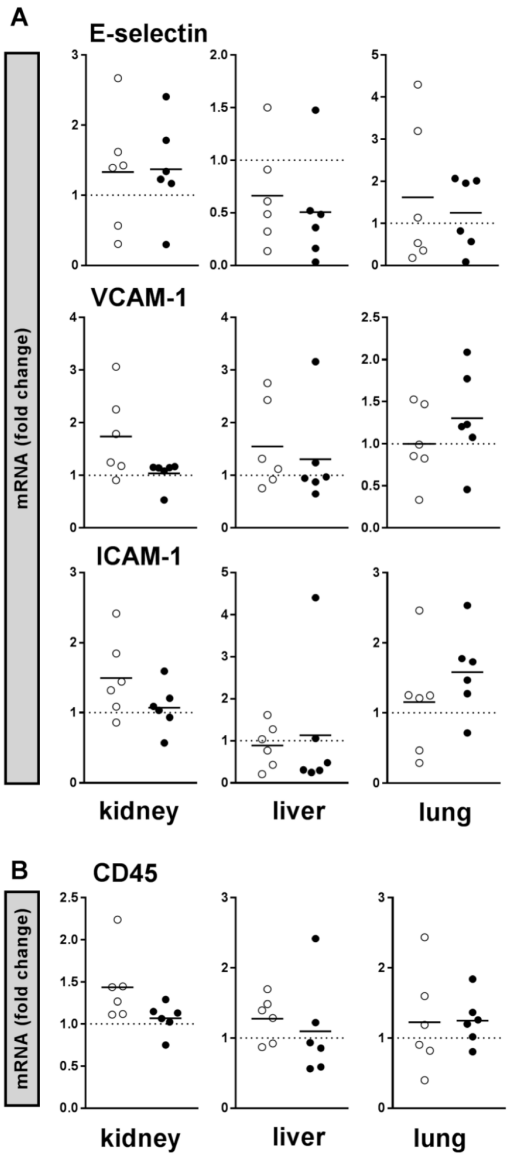



**FIGURE 3. Expression on endothelial inflammatory responses to hemorrhagic shock and resuscitation in kidney, liver, and lung did not differ between Tie2$^{+/+}$ and Tie2$^{+/-}$ mice.** Tie2$^{+/+}$ and Tie2$^{+/-}$ mice were subjected to hemorrhagic shock and resuscitation and sacrificed 1h after resuscitation (HS + R). Organs were assessed for mRNA levels. **A.** E-selectin, VCAM-1, and ICAM-1 mRNA levels. **B.** CD45 mRNA levels. Data are presented as fold change between mice subjected to HS + R and sham (set at 1, ---). Dots represent individual Tie2$^{+/+}$ mice (o), Tie2$^{+/-}$ mice (●), horizontal lines indicate average values of 6 mice per group.

## Endothelial responses to LPS in organs of Tie2$^{+/-}$ mice

As a second model of critical illness, we used LPS to induce endotoxemia to investigate whether partial deletion of Tie2 affected the expression of genes related to endothelial activation [19]. We observed downregulation of Tie2 mRNA and protein in kidney, liver, lung, heart, brain, and intestine after LPS challenge in Tie2$^{+/+}$ mice, which confirmed previous data [12]. In Tie2$^{+/-}$ mice, in which Tie2 expression was already reduced by 50% prior to LPS administration, Tie2 mRNA was additionally downregulated in all organs after LPS administration. The extent of downregulation of Tie2 mRNA was not different between Tie2$^{+/-}$ and Tie2$^{+/+}$ mice in any of the organs analyzed (Suppl. Figure 4).

Next, we studied the effect of LPS administration on the expression of the endothelial adhesion molecules. In all analyzed organs of both Tie2$^{+/-}$ and Tie2$^{+/+}$ mice, mRNA levels of E-selectin, VCAM-1, and ICAM-1 were increased after LPS administration compared to vehicle control (Figure 4A). Interestingly, the induction of expression of E-selectin and VCAM-1 was attenuated in kidney and liver, and of ICAM-1 in the liver, of LPS-treated Tie2$^{+/-}$ mice compared to Tie2$^{+/+}$ mice.

Since we observed an attenuated induction of endothelial adhesion molecule expression in kidney and liver of Tie2$^{+/-}$ mice, we next investigated whether it affected leukocyte infiltration. mRNA expression of the leukocyte marker CD45 was increased in all organs after LPS exposure compared to vehicle control, irrespective of genotype (Figure 4B). However, in LPS-challenged Tie2$^{+/-}$ mice, CD45 mRNA expression was also attenuated compared to its levels in Tie2$^{+/+}$ mice. This effect that was restricted to the kidney.

In summary, 50% reduction in Tie2 protein expression prior to challenge with LPS diminishes upregulation of inflammatory microvascular endothelial responses in an organ-specific way.

## Microvascular bed-specific responses to LPS in Tie2$^{+/-}$ mice

After observing lower adhesion molecule expression in kidney and liver of Tie2$^{+/-}$ mice, we asked the question whether the diminished endothelial inflammatory response to LPS was associated with specific microvascular beds. To this end, we immunohistochemically detected E-selectin and VCAM-1 protein in kidney and liver sections of both mouse lines.

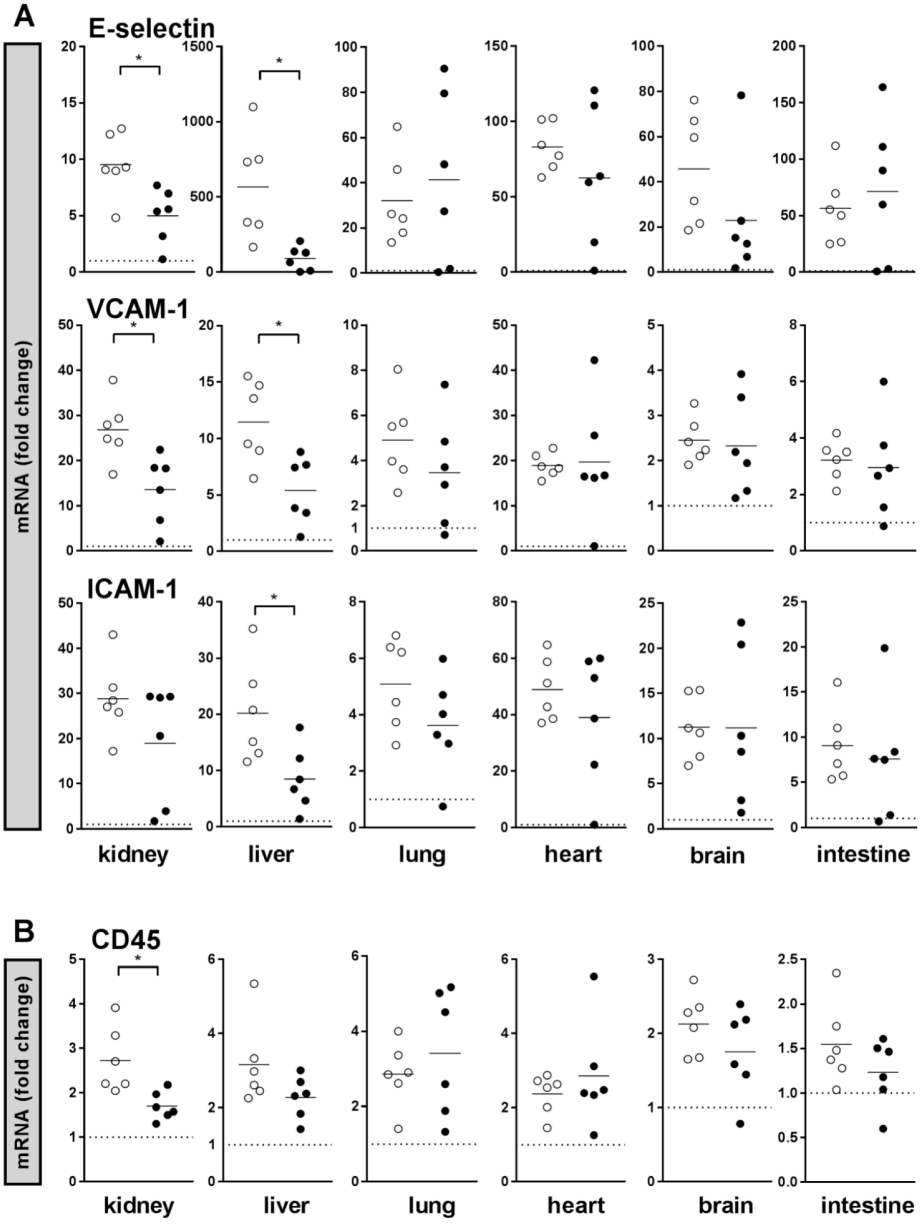
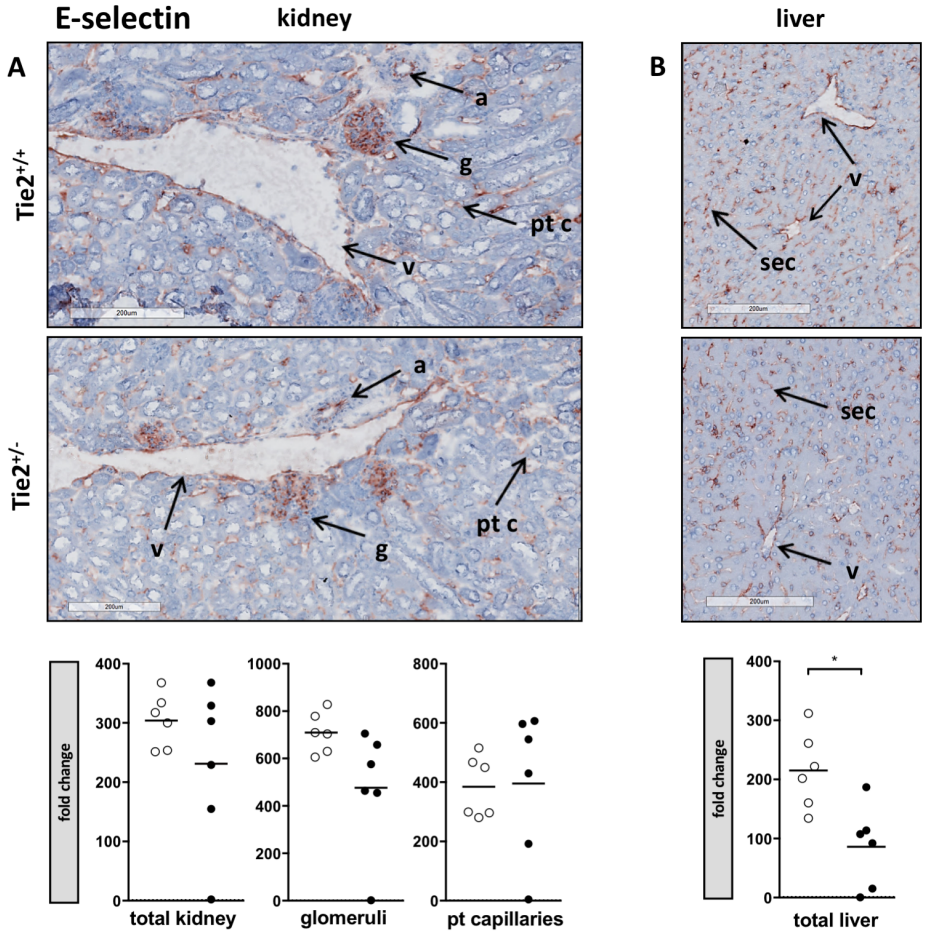



**FIGURE 4. Tie2$^{+/-}$ mice showed diminished inflammatory responses of endothelial cells in distinct organs in response to LPS challenge.** Tie2$^{+/+}$ and Tie2$^{+/-}$ mice were challenged with LPS *i.p.* (1 µg/g) and sacrificed 4h later. Organs were assessed for mRNA. **A.** E-selectin, VCAM-1, and ICAM-1 mRNA levels **B.** CD45 mRNA levels. Data are presented as fold change between LPS treated mice and vehicle control (set at 1, ---). Dots represent individual Tie2$^{+/+}$ mice (o), Tie2$^{+/-}$ mice (●), horizontal lines indicate average values of 6 mice per group, * $P$<0.05.

In kidney and liver of untreated mice of either genotype, E-selectin protein was not expressed in any microvascular segment (data not shown). After LPS exposure E-selectin expression was visible in all microvascular beds in the kidney of both Tie2$^{+/-}$ and Tie2$^{+/+}$ mice, with highest expression in glomeruli and lowest in the peritubular capillaries (Figure 5A). Planimetric quantification revealed no differences in E-selectin protein expression in the different microvascular beds of the kidney between Tie2$^{+/-}$ and Tie2$^{+/+}$ mice (Figure 5A, lower panel). In the liver of both groups, strong E-selectin expression was observed in the sinusoidal capillaries and the venules in response to LPS challenge (Figure 5B). In sinusoidal capillaries of Tie2$^{+/-}$



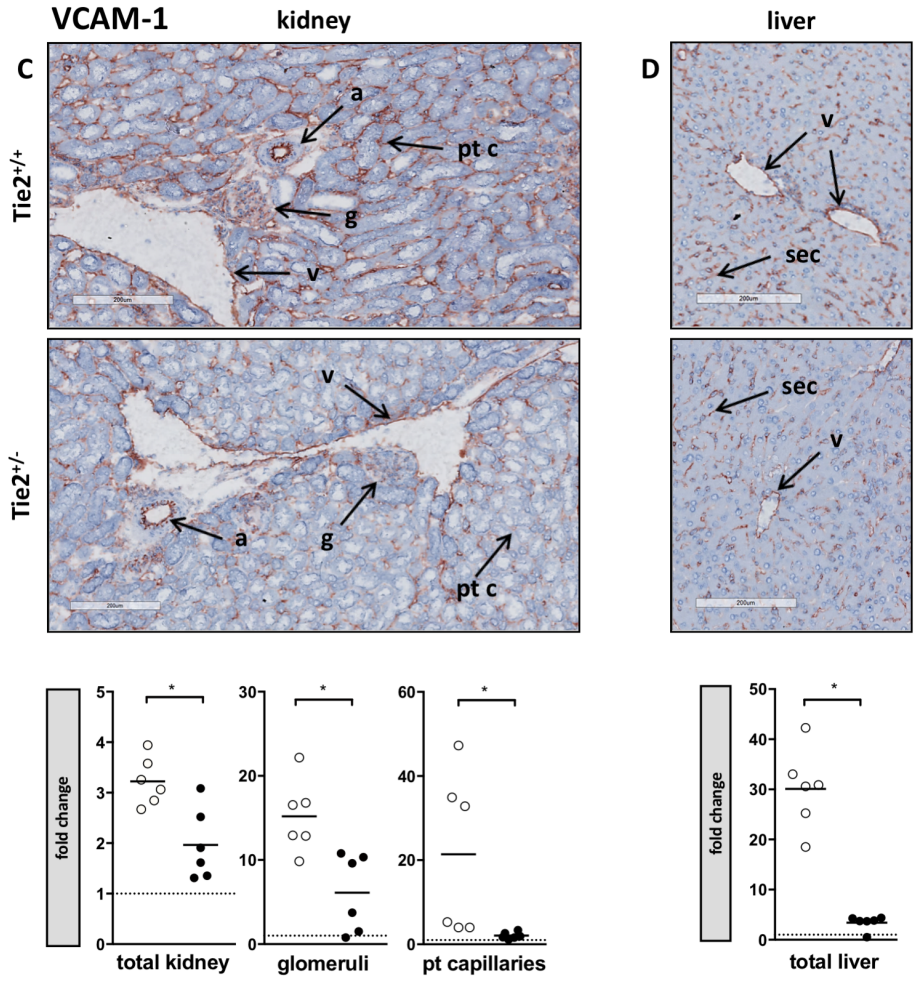



**FIGURE 5. Tie2$^{+/-}$ mice showed diminished inflammatory responses of endothelial cells in an organ and microvascular bed-specific way in response to LPS challenge.** Tie2$^{+/+}$ and Tie2$^{+/-}$ mice were challenged with LPS *i.p.* (1 µg/g) and sacrificed 4h later. Organs were assessed for protein expression by immunohistochemistry. **A** and **B.** Photomicrographs of cryosections of kidney (A) and liver (B) stained for E-selectin, and semiquantitative analysis of E-selectin expression in different microvascular segments by digital planimetry. **C** and **D.** Photomicrographs of cryosections of kidney (C) and liver (D) stained for VCAM-1, and semiquantitative analysis of VCAM-1 expression in different microvascular segments by digital planimetry. Arrows indicate arterioles (a), glomeruli (g), peritubular capillaries (pt c), venules (v), and sinusoidal capillaries (sec). Scale bars 200 µm. Data are presented as fold change between LPS treated mice and vehicle control (set at 1, ---). Dots represent individual Tie2$^{+/+}$ mice (o), Tie2$^{+/-}$ mice (●), horizontal lines indicate average values of 6 mice per group, * $P$<0.05.

mice, E-selectin expression was diminished compared to its levels in their littermate controls. Planimetric quantification of the liver was restricted to total liver, and revealed diminished expression of E-selectin in heterozygous Tie2$^{+/-}$ mice compared to *wild type* Tie2$^{+/+}$ mice.

In untreated mice, VCAM-1 was expressed in all microvascular beds in the kidney and liver. In the kidney, the highest VCAM-1 expression was observed in arterioles, and the lowest expression in glomeruli. In the liver, the extent of VCAM-1 expression was similar in sinusoidal capillaries and venules as microscopically assessed by eye (data not shown). In the kidney of Tie2$^{+/+}$ mice, LPS exposure elicited increased VCAM-1 expression in glomeruli, peritubular capillaries and venules, while in arterioles its expression remained high (Figure 5C). Possibly, additionally induced expression in this particular microvascular segment was masked by already high expression under control conditions. In Tie2$^{+/-}$ mice, LPS treatment led to increased VCAM-1 expression as well, yet the extent of expression was lower in glomeruli, peritubular capillaries and venules was lower to that in Tie2$^{+/+}$ mice. This was confirmed by planimetric analysis (Figure 5C, lower panel). In the liver, LPS-induced expression of VCAM-1 was observed in sinusoidal capillaries and in venules in both Tie2$^{+/-}$ and Tie2$^{+/+}$ mice (Figure 5D). Compared to Tie2$^{+/+}$ mice, lower VCAM-1 expression was mostly observed in the sinusoidal capillaries of Tie2$^{+/-}$ mice. Planimetric analysis of total liver revealed lower VCAM-1 expression in Tie2$^{+/-}$ mice compared to Tie2$^{+/+}$ mice (Figure 5D, lower panel).

To summarize, in kidney and liver pre-existent lower Tie2 levels in the Tie2$^{+/-}$ mice were associated with attenuated microvascular bed-specific expression of E-selectin and VCAM-1 after LPS exposure, implying a role for Tie2 in regulating endothelial cell responses depending on their location in the body.

## Location of leukocyte influx in kidney and liver in response to LPS in Tie2$^{+/-}$ mice

Since endothelial inflammatory adhesion molecules have a prominent role in leukocyte recruitment, we next investigated the effects of diminished expression on localization of infiltrating CD45$^{+}$ leukocytes in kidney and liver of the *wild type* and heterozygous Tie2$^{+/-}$ mice after LPS challenge. In control Tie2$^{+/+}$ and Tie2$^{+/-}$ mice, the many CD45$^{+}$ cells were localized in renal peritubular capillaries, while some were visible in glomeruli (data

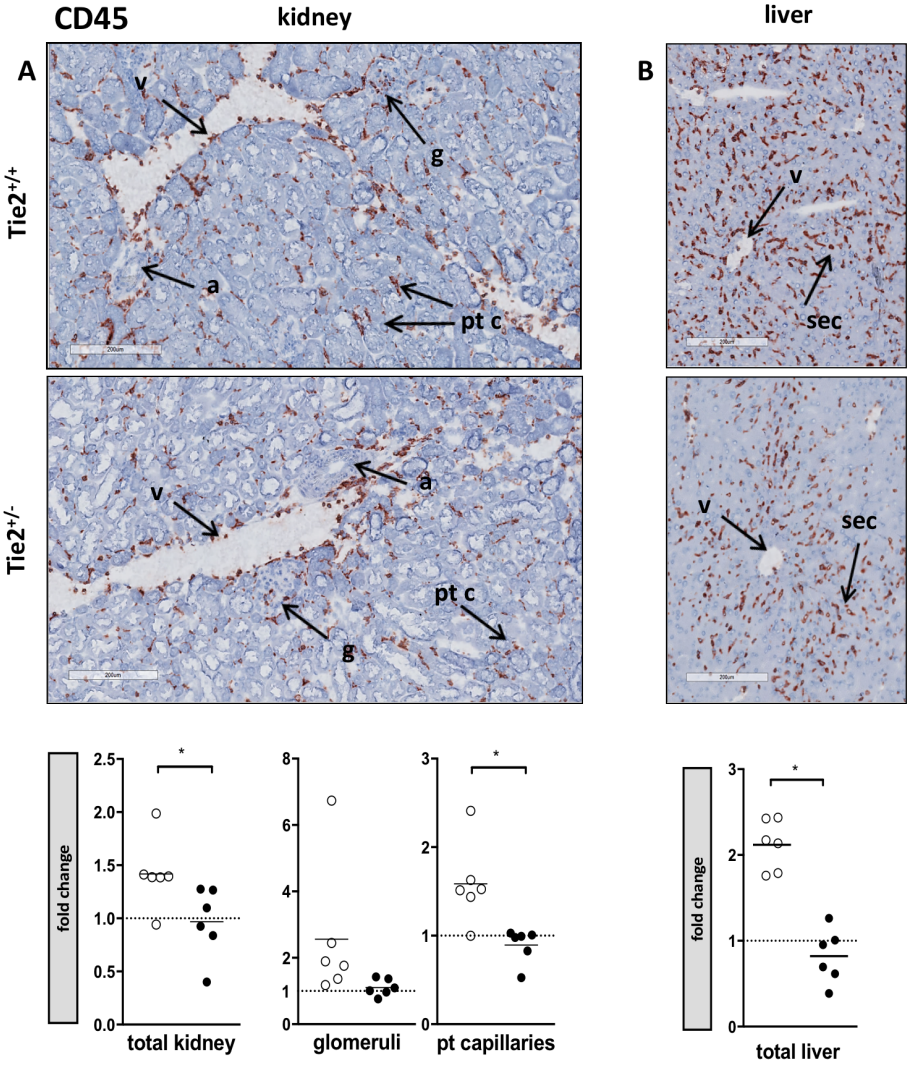



**FIGURE 6. Tie2$^{+/-}$ mice showed diminished leukocyte influx in kidney and liver in response to LPS challenge.** Tie2$^{+/+}$ and Tie2$^{+/-}$ mice were challenged with LPS *i.p.* (1 µg/g) and sacrificed 4h later. Organs were assessed for protein expression by immunohistochemistry. **A** and **B.** Photomicrographs of cryosections of kidney (A) and liver (B) stained for CD45$^{+}$ leukocytes, and semiquantitative analysis of CD45$^{+}$ cells in different microvascular segments by digital planimetry. Arrows indicate positive (red) cells in microvascular structures; arterioles (a), glomeruli (g), peritubular capillaries (pt c), venules (v), and sinusoidal capillaries (sec). Scale bars 200 µm. Data are presented as fold change between LPS treated mice and vehicle control (set at 1, ---). Dots represent individual Tie2$^{+/+}$ mice (o), Tie2$^{+/-}$ mice (●), horizontal lines indicate average values of 6 mice per group, * $P$<0.05.

not shown). After LPS administration, increased numbers of CD45$^{+}$ cells localized in glomeruli and in the peritubular capillaries of the kidney in both Tie2$^{+/-}$ and Tie2$^{+/+}$ mice compared to vehicle controls (Figure 6A). Compared to *wild type* mice, lower numbers of CD45$^{+}$ cells were observed in the renal peritubular capillaries in Tie2$^{+/-}$ mice. Planimetric quantification supported this observation (Figure 6A, lower panel). In the liver of control Tie2$^{+/+}$ and Tie2$^{+/-}$ mice, scattered CD45$^{+}$ cells were localized mainly in sinusoidal capillaries (data not shown). After LPS exposure, increased numbers of leukocytes were observed in sinusoidal capillaries in both Tie2$^{+/-}$ and Tie2$^{+/+}$ mice (Figure 6B). Reduced numbers of CD45$^{+}$ cells had accumulated in the sinusoidal capillaries of Tie2$^{+/-}$ mice compared to Tie2$^{+/+}$ mice. Planimetric quantification of the total liver confirmed reduced CD45$^{+}$ cell localization in the liver of Tie2$^{+/-}$ mice compared to littermate controls (Figure 6B, lower panel).

To summarize, lower Tie2 levels as present in Tie2$^{+/-}$ mice were associated with reduced numbers of leukocytes infiltrating in kidney and liver after LPS exposure, which is likely a consequence of the attenuated local expression of inflammatory endothelial adhesion molecules.

## Discussion

Tie2 is a receptor tyrosine kinase that is mainly expressed by blood vessel endothelial cells and plays a role in vascular integrity and inflammatory responses. Tie2 mRNA and protein levels are decreased in critical illness [12, 20]. While its ligands Ang1 and Ang2 have been extensively studied with regard to their spatiotemporal changes in expression and functional consequences thereof in response to inflammatory processes, functional consequences of reduced Tie2 levels on inflammatory endothelial responses in the microvasculature in organs are unknown. This study was designed to investigate effects of reduced Tie2 presence on the inflammatory responses of endothelial cells in the microvasculature in organs of mice in health and in critical illness models. In a newly generated heterozygous Tie2$^{+/-}$ mouse model in which deletion of exon 9 in one allele of the Tie2 gene resulted in 50% reduction of Tie2 expression, we showed that this loss did not affect basal expression levels of the Tie2 ligands Ang1 and Ang2, nor of endothelial inflammatory genes E-selectin, VCAM-1, and ICAM-1. We did not

observe differences in inflammatory gene expression related to endothelial activation and leukocyte influx between $Tie2^{+/+}$ and $Tie2^{+/-}$ mice exposed to HS + R, LPS exposure on the other hand did reveal an attenuated response in mice expressing 50% less Tie2. This attenuated inflammatory response was restricted to the microvasculature of kidney and liver, and were shown to be microvascular bed and gene-specific.

Our new Tie2 mutant mouse line corroborates several findings in a previous Tie2 mutant mouse, generated by Dumont et al., in which exon 1 of the Tie2 gene was deleted ($\Delta E1/Tie2^{+/}$)[21]. Firstly, no homozygous Tie2 knockout mice were born in our Tie2 mutant line, which is in agreement with Dumont's observation that Tie2 homozygous knockout mice had embryonically lethal vascular malformations [21]. Second, deletion of one Tie2 allele in our model did not affect basal expression of the Tie2 ligands Ang1 and Ang2, nor that of endothelial adhesion molecules in any of the five organs studied. This complements previous data published by Ghosh *et al*, using the aforementioned $\Delta E1/Tie2^{+/-}$ mice and showed similar results on Ang1 and Ang2 expression in the lungs of $Tie2^{+/-}$ mice [22].While Ghosh *et al.* focused on lung, our study is the first to report no changes in basal Ang1 and Ang2 expression levels in multiple individual organs of adult heterozygous $Tie2^{+/-}$ mice while experiencing lower Tie2 expression levels starting as early as in embryo. These finding in $Tie2^{+/-}$ mice indicate that adaptation to normalized expression levels of Ang1 and Ang2 to the lower Tie2 levels is not required for maintenance of vascular integrity in the adult microvasculature.

As previously reported, LPS administration suppresses Tie2 expression [12] and at the same time it induces activation of the NF-κB pathway, leading to a pro-inflammatory endothelial response in mouse organs [8, 23]. Our data on the absence of effects of lower Tie2 expression on inflammatory endothelial cell reaction to LPS in the lung support the findings by Ghosh *et al.* [22], who also did not observe differences in adhesion molecule expression in the lung of $Tie2^{+/-}$ mice that received 15 mg/kg *i.p.* LPS when compared to *wild type* controls. In contrast, McCarter *et al.* reported in $\Delta E1/Tie2^{+/-}$ mouse model reduced expression of E-selectin and VCAM-1 protein in lung compared to controls after intra tracheal instillation of LPS at 800 µg dose [24]. A possible explanation for the discrepancy between McCarter's findings and those of Ghosh and ours could be that intra tracheal instillation of LPS leads to higher local LPS levels than when administered

intraperitoneally. Whether higher *i.p.* or intra tracheally applied doses of LPS administered to our Tie2$^{+/-}$ mice would unmask Tie2 expression related differences in adhesion molecule expression in lung needs to be established.

An important finding in our study is that a 50% reduction in Tie2 protein has functional consequences for particular micro vessels in the body, while not affecting others. The molecular mechanism(s) behind this phenomenon is (are) unclear at present. It is known that after LPS administration, Ang2 is released from endothelial Weibel-Palade bodies [3, 25] and can then compete with Ang1 for binding to Tie2, thereby inhibiting Tie2 phosphorylation [26]. As a consequence, the NF-κB pathway is inhibited [27] and expression of pro-inflammatory genes is suppressed. In our LPS-treated Tie2$^{+/-}$ heterozygous mice, an attenuated expression of Ang2 was observed in liver and lung (Suppl. Figure 5). A reduced influx of neutrophils in response to *i.p* injection of bacteria was shown in the peritoneal cavity in Ang2 knockout mice [9] and in the lungs of mice where Ang2 was blocked with specific antibodies [10]. In mice where binding of Ang2 to its receptor was prevented using a specific inhibitor, reduced expression of ICAM-1 and VCAM-1 was shown [28]. Both studies are indicative of a relation between the Ang/Tie2 system and the NF-κB pathway. Furthermore, using laser dissection microscopy to isolate microvascular segments from kidneys of mice [29] prior to gene expression analysis, we found that each microvascular segment has its own Ang1/Ang2/Tie2 expression profile (unpublished data). Similarly, other endothelial cell controlling molecular systems such as VEGF and its receptors are heterogenically expressed in the renal microvascular segments [20]. How this links to the microvascular segment specific responses in the absence of Tie2 expression as shown here, remains elusive to date. Studying the phosphorylation status of Tie2 in the different organs and microvascular segments of Tie2$^{+/-}$ mice as well as NF-κB nuclear translocation in time in response to LPS in both *wild type* and Tie2$^{+/-}$ mice could shed light on this.

The dependence of endothelial cell responses to an inflammatory stimulus on Tie2 in particular microvascular beds were only observed in the endotoxemia model, not in the hemorrhagic shock and resuscitation model. In this latter model, we observed a wide variation in microvascular responses in the HS + R groups as well as in sham groups of both genotypes, so our study may have been underpowered to detect small differences in endothelial behavior induced by HS + R in combination with the lower expression of

Tie2. Furthermore, the installation of the anesthesia and instrumentation procedure by itself already induces inflammatory responses [13] and is a confounding factor that may hamper identification of small differences between wild type and transgenic mice in this critical illness model

To summarize, we here demonstrate that deletion of exon 9 in one allele of the Tie2 gene results in a mouse line that expresses 50% less Tie2. While partial deletion of Tie2 had no significant effect on microvascular responses in HS + R as a mild model of critical illness, after LPS administration lower Tie2 expression was associated with reduced endothelial responses in kidney and liver. These responses were restricted to particular microvascular beds in these organs, and were paralleled by changes in leukocyte recruitment. These data indicate that Tie2 has different functions in controlling endothelial cell behavior depending on their location in the body.

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# Supplementary Materials and Methods

### RNA Isolation of white blood cells from whole blood

Whole blood was kept on ice until further handling and centrifuged for 10 min at 14,000g and 4°C. Plasma was removed and red blood cells were lysed twice with 10 ml ice-cold $NH_4Cl$. Cells were pelleted by centrifugation and the pellet was resuspended in Trizol (Invitrogen, Breda, The Netherlands). RNA was isolated according to standard protocols.

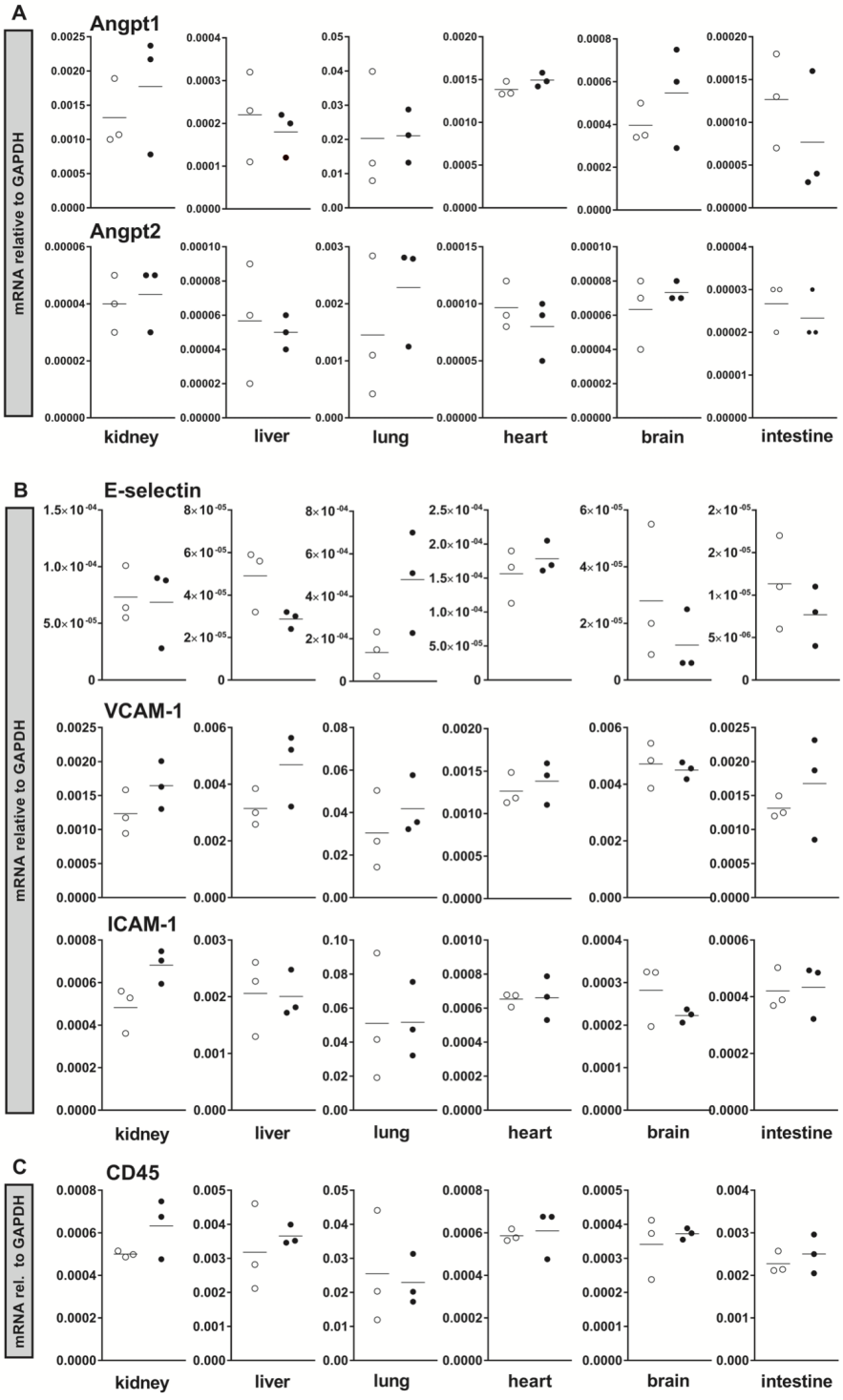
# Supplementary Table

**SUPPLEMENTARY TABLE 1. CT values of Gapdh, Ptprc, and Tek in white blood cells of individual Tie2$^{+/+}$ and Tie2$^{+/-}$ mice as assessed with RT-qPCR.**

| Gene | Tie2$^{+/+}$ (n=3) | | | Tie2$^{+/-}$ (n=4) | | | |
|---|---|---|---|---|---|---|---|
| *Gapdh* | 23.9 | 23.5 | 21.9 | 24.8 | 22.8 | 23.7 | 24.4 |
| *Ptprc* | 26.1 | 26.2 | 24.9 | 27.2 | 24.4 | 27.0 | 28.4 |
| *Tek* | 37.4 | 36.7 | 36.6 | >40 | 36.8 | 36.3 | >40 |

Gapdh: glyceraldehyde 3-phosphate dehydrogenase; Ptprc: protein tyrosine phosphatase, receptor type C, CD45; Tek: tyrosine kinase, Tie2

**SUPPLEMENTARY FIGURE 1. Basal mRNA expression levels of Tie2 ligands Angpt1 and Angpt2, endothelial inflammatory adhesion molecules, and surveilling leukocytes in organs of Tie2$^{+/+}$ and Tie2$^{+/-}$ mice.** Organs of Tie2$^{+/+}$ and Tie2$^{+/-}$ mice were assessed for mRNA levels by RT-qPCR relative to GAPDH. **A.** Ang1 and Ang2 mRNA levels. **B**. E-selectin, VCAM-1, and ICAM-1 mRNA levels. **C.** CD45 mRNA levels. Dots represent individual Tie2$^{+/+}$ mice (o), Tie2$^{+/-}$ mice (●), horizontal lines indicate average values of 3 mice per group.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Angpt1
Angpt2
mRNA relative to GAPDH
kidney
liver
lung
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B
E-selectin
VCAM-1
ICAM-1
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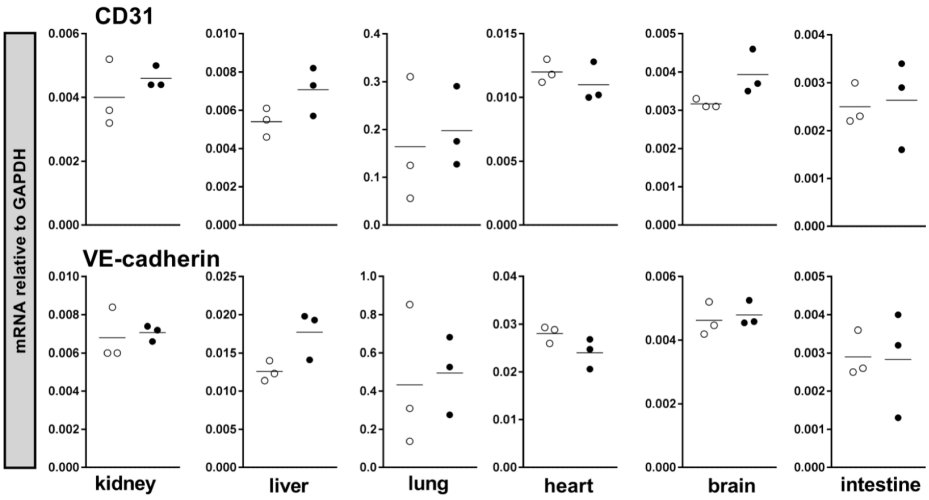
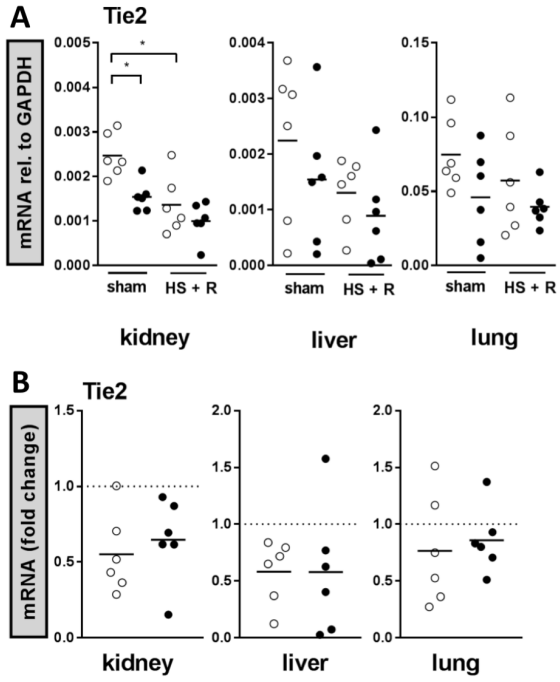
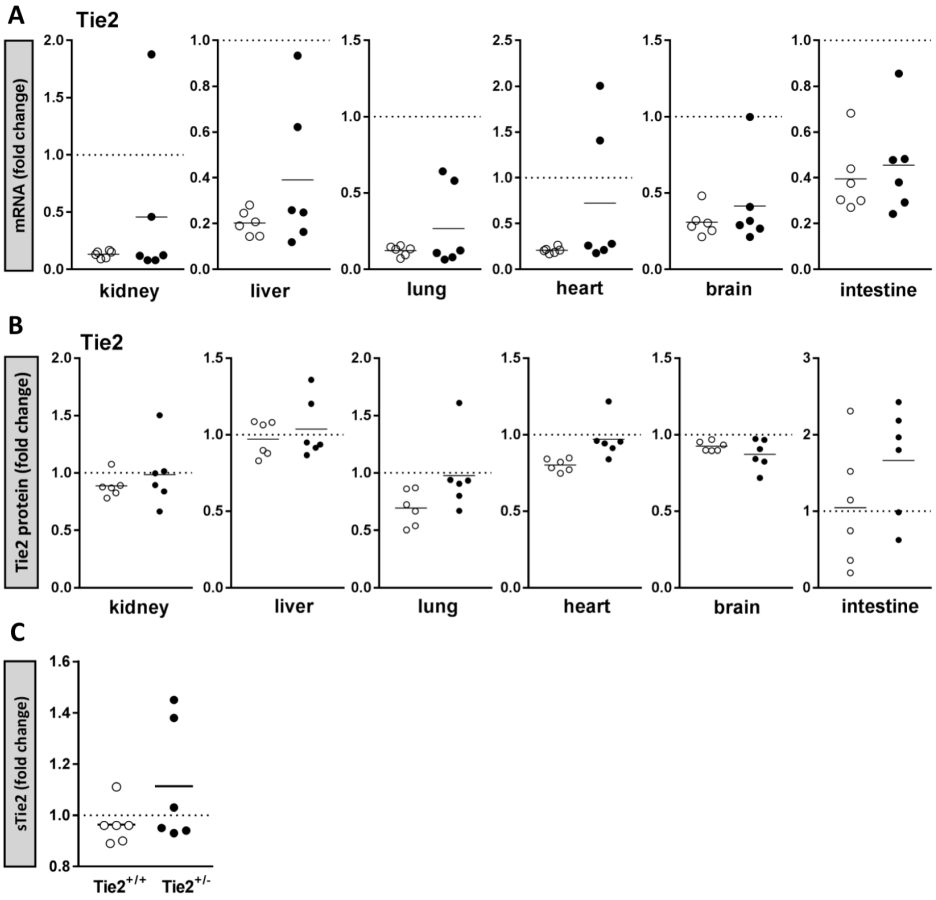


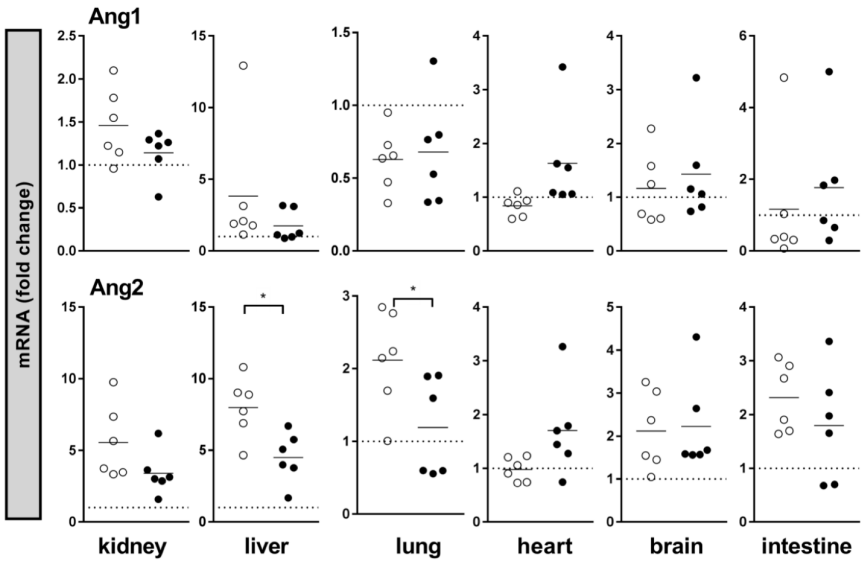

**SUPPLEMENTARY FIGURE 2. Basal expression levels of pan-endothelial genes Pecam1 and Cdh5 in organs of Tie2$^{+/+}$ and Tie2$^{+/-}$ mice.** Organs of Tie2$^{+/+}$ and Tie2$^{+/-}$ mice were assessed for mRNA levels by RT-qPCR relative to GAPDH. A. Pecam1 mRNA levels. B. Cdh5 mRNA levels. Dots represent individual Tie2$^{+/+}$ mice (○), Tie2$^{+/-}$ mice (●), horizontal lines indicate average values of 3 mice per group.




**SUPPLEMENTARY FIGURE 3. Effects of hemorrhagic shock and resuscitation on Tie2 mRNA expression in kidney, liver, and lung in Tie2$^{+/+}$ and Tie2$^{+/-}$ mice.** Tie2$^{+/+}$ and Tie2$^{+/-}$ mice were subjected to hemorrhagic shock and resuscitation and sacrificed 1h after resuscitation (HS + R). Kidney, liver, and lungs were assessed for Tie2 mRNA levels relative to GAPDH **(A)** and as fold change **(B)** between HS + R and sham (set at 1, ----). Dots represent individual Tie2$^{+/+}$ mice (o), Tie2$^{+/-}$ mice (●), horizontal lines indicate average values of 6 mice per group, * $P$<0.05.




**SUPPLEMENTARY FIGURE 4. Effects of LPS challenge on Tie2 expression in organs of Tie2$^{+/+}$ and Tie2$^{+/-}$ mice.** Tie2$^{+/+}$ and Tie2$^{+/-}$ mice were challenged with LPS *i.p.* (1 µg/g) and sacrificed 4h later. **A.** Tie2 mRNA levels in organs. **B.** Tie2 protein expression in organs. **C.** soluble Tie2 (sTie2) levels in plasma. Data are presented as fold change between LPS treated mice and vehicle control (set at 1, ----). Dots represent individual Tie2$^{+/+}$ mice (o), Tie2$^{+/-}$ mice (●), horizontal lines indicate average values of 6 mice per group.




**SUPPLEMENTARY FIGURE 5. Expression levels of Angpt1 and Angpt2 in organs of Tie2$^{+/+}$ and Tie2$^{+/-}$ mice after LPS challenge.** Tie2$^{+/+}$ and Tie2$^{+/-}$ mice were challenged with LPS *i.p.* (1 µg/g) and sacrificed 4h later. Organs were assessed for mRNA levels of Angpt1 and Angpt2. Data are presented as fold change between LPS treated mice and vehicle control (set at 1, ---). Dots represent individual Tie2$^{+/+}$ mice (o), Tie2$^{+/-}$ mice (●), horizontal lines indicate average values of 6 mice per group, * $P$<0.05.